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INFECTION OF MAN WITH BACTERIUM TULARENSE *

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PLATE 3

We believe this to be the first recorded instance of human infection with the organism discovered by McCoy and Chapin in a plague-like disease first described by McCoy in the California ground-squirrel (*Citellus beecheyi* Richardson).[1] In our work we have added very little that is new connected with the biology of the organism, besides the fact that our virus came from a human being who in all probability contracted it while dressing meats for a restaurant. In this connection we might remark that about a year previously we had heard from a hunter that wild rabbits were dying in large numbers across the Ohio River in Kentucky. We did not see any of these rodents, and by the time we had worked out the etiology of our case it was too late to investigate wild game sold in the markets. For the fact that our own work was carried out to a satisfactory conclusion in a relatively short time, we are indebted to the work of McCoy and Chapin, whose labors covered a considerable period; for we did not succeed in cultivating the virus until we used the coagulated egg-yolk recommended by them. While we occasionally refer to some of the extensive experiments of these workers, we do not review them in detail, as the publications referred to are easily obtained.

HISTORY OF CASE[2]

On Nov. 24, 1913, the patient, E. E., a man of 21 years, who was meat cutter in a restaurant, came, presenting an inflammation of the left eye which he said started on November 21. On examination about ten discrete ulcers, varying from 1 to 4 mm. in diameter, were seen on the conjunctiva covering the upper and lower tarsi. The lids were edematous. The preauricular gland on the left side was swollen and tender. During the next three days the edema increased, the

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* Received for publication, June 6, 1914.

[1] *Public Health Bull.* 43, *U. S. Public Health Service,* Washington, 1911; *ibid.,* No. 53, 1912; and *Jour. Infect. Dis.,* 1912, 10, p. 61.

[2] We are indebted to Dr. Derrick T. Vail of Cincinnati for the greater part of this clinical history.

ulceration became a little more marked, and the lymphatic glands in the left anterior triangle of the neck and the left submaxillary glands became tender and swollen. On November 28, a week after the onset, the patient's temperature was 101.6 F.; he looked cachectic and complained of losing weight. A discrete pustular eruption now appeared on the left temporal and malar region. Glanders was suspected and the patient was sent to the Cincinnati hospital. By December 1, in addition to the above symptoms, which persisted, the inflammatory process spread to the left lacrimal sac and resulted in abscess formation. At this time smears and cultures on various media were made. No bacteria could be found microscopically, nor did any growth appear on the culture media during about a month's observation. Scrapings from one of the ulcers were injected into a male guinea-pig as follows:

Guinea-Pig 1.—Received on Dec. 4, 1913, an intraperitoneal injection of scrapings from a conjunctival ulcer suspended in sterile physiologic salt solution. Died, December 10. Kept on ice over night. Necropsy, December 11: The body is frozen solid. On section the subcutis and superficial glands appear normal. The lungs show acute pneumonia, the spleen and liver are congested and enlarged, and show numerous scattered foci of necrosis. Smears from the heart's blood and various organs were stained by Giemsa's method, with Löffler's methylene-blue, by Gram's and by the tubercle method, and careful search failed to reveal anything looking like bacteria excepting that in the Giemsa preparations very minute scattered coccoid granules were seen. Both aerobic and anaerobic cultures made on a variety of media remained sterile at 37 C. and at room temperature during a month's observation.

The patient left the hospital still suffering from the disease, and further efforts to get material from him for confirmatory inoculations failed. There can hardly be any doubt, however, about the virus coming from the human case, since we have been able to reproduce the gross changes in the conjunctivae so accurately in experimental animals.

ISOLATION OF THE VIRUS

The disease was kept going through a series of guinea-pigs while experiments on isolation were in progress. Aerobic and anaerobic cultures were made in various media, e. g., + 1 agar; agar with glycerin; with glucose, maltose and other sugars; with human blood, ascites fluid, defibrinated rabbit's, rat's and guinea-pig's blood; Löffler's blood-serum; in + 1 broth containing sterile pieces of guinea-pig's liver, spleen and kidney (with and without a covering of oil). In all instances the cultures remained sterile excepting that once a bacillus with a tendency to produce capsules, and another time a diplococcus, was grown. Neither of these was pathogenic for guinea-pigs. All the cultures were kept under observation for about a month before they were discarded. The following inoculations show that the virus did not live for long in some of these cultures.

Guinea-Pig 16.—Inoculated subcutaneously with 1 c.c. of a forty-eight-hour culture (37 C.) from White Rat 4 — made by placing one-half of its spleen in a tube of + 1 broth containing a piece of fresh guinea-pig's liver and covering the surface with sterile olive oil. A minute streptococcus was present in the culture. The guinea-pig appeared healthy for six days, but was chloroformed in a dying condition on the seventh. No signs of the disease were present and cultures from its heart's blood remained sterile.

Guinea-Pig 18.—Inoculated subcutaneously with 1.5 c.c. of a six-day-culture (37 C.) from Rabbit 1 — made by placing a bit of the rabbit's spleen in + 1 broth containing a fresh piece of the kidney of a guinea-pig and covering with sterile olive oil. The guinea-pig remained well during twenty days' observation, when it was reused successfully for a passage of the virus.

After we had passed the virus through twenty-four animals, we became acquainted with the work of McCoy and Chapin, and by using the coagulated egg-yolk on which they were able to grow *Bacillus tularense* we isolated what we believe to be the same bacterium.

During these attempts at cultivation we made careful studies of fresh preparations and smears from the organs of dead and freshly killed animals. The smears were fixed by heat, with methyl alcohol, with Zenker's or Schaudinn's fluid and stained over short and long intervals with Löffler's methylene-blue or carbol fuchsin, with Giemsa's and McNeal's stains, by Gram's method and aqueous safranin and with Heidenhain's iron-alum hematoxylin. Vital staining with various anilin dyes was tried. The preparations were studied carefully with apochromatic lenses and compensating oculars without revealing anything we could identify as a probable micro-organism. The following two filtration experiments showed that the virus would not pass a Berkefeld filter.

(*a*) Guinea-Pig 4 was chloroformed when dying and 0.5 c.c. of its viscous peritoneal exudate was diluted with 20 c.c. of 0.85 per cent sodium chlorid solution and filtered through a new No. 5 Berkefeld filter. Guinea-Pig 6, 380 gm. was inoculated intraperitoneally with 10 c.c. of the filtrate. It remained well for twenty-three days, when it died for some unknown reason.

(*b*) The enlarged speckled spleen of Rabbit 3, dead of the disease, was kept on ice in a sterile Petri dish for forty-eight hours. It was examined and found microscopically sterile. Half of the spleen was ground up in a mortar and suspended in 40 c.c. of sterile 0.85 per cent sodium chlorid solution. After sedimenting for one-half hour, the supernatant fluid was filtered through the same No. 5 Berkefeld filter used in (*a*). The filter was boiled thoroughly in sodium carbonate solution, washed with distilled water until the filtrate was no longer alkaline to phenolphthalein, and then sterilized in the autoclave. It took fifteen minutes at ordinary pressure to filter 20 c.c. of perfectly clear, straw-colored fluid. Guinea-Pig 19, 290 gm., inoculated intraperitoneally with 6 c.c. of this filtrate remained well for nine days, when it was found dying with spasmodic convulsions. On dissection it showed none of the lesions of the disease and another guinea-pig (No. 26, 350 gm.) inoculated intraperitoneally with 2 c.c. of its peritoneal exudate and heart's blood remained well for nineteen days, when it was killed, dissected and found normal.

Guinea-Pig 20 (365 gm.), used as a control, was injected subcutaneously with 2 c.c. of the unfiltered extract. It died in three days with typical lesions, and *B. tularense* was isolated in an ovomucoid-yolk culture from an inguinal bubo.

These experiments agree with those of McCoy and Chapin. Since the virus would not pass the Berkefeld No. 5, which allows some clearly visible bacteria to go through, as was shown by one of us in repeating some work done previously by Theobald Smith on the bacillus of guinea-pig pneumonia,[3] we felt that probably it was not ultramicroscopic. The virus described below is just as small as, if not smaller than, that of guinea-pig pneumonia, so that its failure to pass the filter may possibly be due to the presence of the large amount of capsular substance.

We examined preparations from the conjunctival ulcers and spleen of an infected rabbit, dead for from six to eight hours, in the dark field. This revealed nothing except the presence of very numerous minute granules with active brownian motion, and similar colloidal granules could be found in smaller numbers in the spleen of a normal rabbit. In all probability some of these granules were *B. tularense* which assumes a coccoidal form post mortem, but they could not be distinguished from other colloidal granules.

DESCRIPTION OF THE VIRUS AND ITS IDENTITY WITH BACILLUS TULARENSE

After we had obtained cultures of *B. tularense* from some of our animals, we found by experimenting that while carbolic acid and caustic potash were inefficient mordants, anilin oil gave excellent results with the cultures and with tissue smears. We have obtained the clearest pictures with anilin-water Hoffman's violet or Victoria blau; anilin-water fuchsin, dahlia and gentian violet also stain the bacilli in smears, but they are not differentiated as clearly; anilin-water nacht blau and toluidin blau failed to stain the bacilli even after fifteen to thirty minutes.

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[3] *Jour. Med. Research,* 1902, 3, p. 322.

As an example of this selective staining we may quote the following:

Guinea-Pig 27 (480 gm.) was autopsied some hours after death and a culture from its spleen on egg-yolk showed a spreading, moist growth which is not characteristic of *B. tularense.* Smears showed enormous numbers of bacilli which stained readily with Löffler's methylene-blue, but when the smears were stained with anilin Hoffman's violet, enormous numbers of minute coccoid bodies were also seen among the rods.

B. tularense does not retain the stain in Gram's method, nor will it take a contrast stain of aqueous safranin in such preparations. While McCoy and Chapin state that it is stained with anilin gentian violet and carbol fuchsin, we have not been able to demonstrate it clearly with the latter dye in tissue smears, although this stain does fairly well for smears from cultures, where, in our experience, the capsular substance is greatly diminished in amount.

It is a minute rod surrounded by a considerable amount of capsular substance when in the tissues of infected animals. We have not tried to stain this capsule in smears, but it is apparent as a very well-defined, unstained halo. In our experience the rod shape is only to be seen when an animal is chloroformed before death. Post mortem, the organism soon rounds up into coccoid bodies, and this latter form is the only one we have seen in coagulated egg-yolk or yolk-ovomucoid cultures. No distinct capsule is to be seen in cultures. When measured under a Zeiss 3 mm. N. A. 1.40, the coccoid form from egg-yolk cultures measures about 0.5 to 0.6 micron in diameter. The rods, including the capsular halo, measured about 1 to 1.5 microns × 0.7 micron. We will not insist on the accuracy of these measurements, however, as an ocular micrometer was used.

McCoy and Chapin give the following approximate measurements for *B. tularense:* length, 0.3 to 0.7 micron; length of capsule, 0.4 to 1 micron; breadth, 0.2 micron; breadth of capsule, 0.3 to 0.5 micron. They also note that coccoid forms occur in the tissues and that irregular and globular forms predominate in cultures.

Cultural Characters.—We have obtained growths from the spleen, liver, buboes and heart's blood of animals on the coagulated hen's egg-yolk, recommended by McCoy and Chapin. We can confirm their observation concerning the importance of having the egg-yolk of just the right consistency. The medium must be of a rubbing consistency, so that it fluctuates when touched, and yet the surface must be firm enough for inoculation. Further solidification beyond this point, as usually practiced in making Dorset's medium, renders the medium unfit for the use of the bacterium. McCoy and Chapin recommend diluting the egg-yolk with 20 per cent. of water or milk. We have taken the yolk of perfectly fresh hen's eggs and tubed it after diluting it with an equal quantity of sterile 0.85 per cent. sodium chlorid solution. The whole procedure is carried out with aseptic precautions. The slanted tubes are then coagulated at from 70 to 90 C., being taken out of the sterilizer when they have attained just the right consistency.

On such slants, inoculated from the tissues of experimental animals and incubated aerobically at 37 C., growth appears on the second or third day. The colonies are very minute at first, but reach a diameter of from 1 to 3 mm. They appear like minute drops of ovomucoid. They are viscous and emulsify with difficulty in salt solution. When removed with precautions to avoid taking up yolk granules from beneath, and examined in hanging drops of 0.85 per cent. sodium chlorid solution or broth, they appear as minute coccoid bodies. We have watched them for hours under the Zeiss 3 mm. N. A. 1.40 with compensating oculars, and feel sure that under these conditions of oxygen tension they show no motility excepting active brownian motion. The coccoid bodies are discrete, and but rarely can one find two which are in juxtaposition. As one might expect, actual fission has not been observed under these circumstances. Egg-yolk cultures have been found viable after three months at 15 C. A culture isolated on January 27 and subcultured every third or fourth day until March 24, was found virulent for Guinea-Pig 49, but produced a subacute form of the disease.

All attempts to accustom this organism to other media, even after several weeks' cultivation on egg-yolk, have failed except that we found that it would grow in the ovomucoid-yolk medium recommended by one of us for the cultivation of amebae.[4] Here the fluid medium showed no visible change except that in three or four days after inoculation with a small piece of an inguinal bubo (guinea-pig) the medium solidified so that the tube could be turned upside down without spilling the culture—just as happens when this medium is inoculated with *Bacillus pyocyaneus*. Evidently something like pseudomucin is produced, and the production of some such substance probably accounts for the viscosity of the colonies and the viscosity of the peritoneal exudates in experimental animals. Subcultures in the same medium gave the same results at 37 C. Here, too, the organism occurs in the coccoid form.

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[4] *Arch. f. Protistenk.*, 1913, 30, p. 77.

Pathogenicity.—We have not experimented on animals as extensively as McCoy and Chapin, but have used forty-nine guinea-pigs, three Belgian hares, three white rats, three kittens and one pigeon. The guinea-pigs, as a rule, succumbed on the fourth or fifth day after cutaneous inoculation with spleen juice or when pricked in the eye with an infected needle. In a series of twelve passages (including one rabbit), where the virus was passed from the spleen of a dead animal to the conjunctiva or cutis of a living one, there was no marked diminution or exaltation of virulence.

As an example of the usual lesions in guinea-pigs, we quote the following protocol of Guinea-Pig 3 (439 gm.) : Inoculated cutaneously by rubbing a fragment of the spleen of Guinea-Pig 2 on a scratch on its abdomen. Three days later the site of inoculation showed an area of congestion. In the center of this area was a yellowish dry, crusty scab beneath which there was no fluid pus. The animal was very sick and weak. It died on the night of the fourth day. At the postmortem next morning the site of inoculation showed a yellowish area of coagulation necrosis, 1 by 2 inches, surrounded by congested vessels which radiated especially to the inguinal glands. These glands were swollen, 3 by 4 mm., were hard and not caseous on section, and were surrounded by intensely congested and edematous tissue. The liver and spleen were swollen and enlarged and speckled with numerous yellowish-white foci of necrosis. The kidneys were intensely congested. The adrenals were swollen and so congested as to appear blackish and were as friable as a clot of blood. The mesenteric glands were swollen and showed a few foci of necrosis on section. The intestines and their follicles appeared normal. The mucous membrane of the stomach appeared normal, but the organ was full of a clear viscous mucus. The epididymi were congested. The vessels of the head were deeply congested and the heart was full of dark-clotted blood. The lungs were congested and showed some small areas of consolidation and a few subserous petechiae. The brain appeared normal to the eye.

The hemorrhagic congestion of the adrenals is not always so marked. The peritoneal cavity often contains a small amount of extremely viscous fluid. Once we noted very marked involvement of the lymph follicles of the small intestine with intestinal subserous hemorrhages in a guinea-pig inoculated cutaneously. It appears to us, on comparing our notes with those of McCoy and Chapin, that we were dealing with a somewhat more virulent virus, as shown by the somewhat shorter course of the disease in our animals and the absence of caseation of the buboes which they note as characteristic.

We kept the virus going chiefly by rubbing a little spleen pulp into a scratch on the abdomen of animals. Simply dipping a fine needle into the spleen of a dead animal or into a culture, and pricking the ocular or palpebral conjunctiva of rabbits or guinea-pigs results in the production of multiple areas of necrosis on the palpebral conjunctivae, just like those in the human case, and is followed by septicemia and death within a very few days.

The rabbits formed an eye inoculation series—No. 2 being inoculated from the spleen of No. 1, and No. 3 from the spleen of No. 2. Death occurred on the sixth, third and fourth days, respectively.

As an example of the lesions in rabbits, we may quote the protocol of Rabbit 1. The post-mortem appearance of the eye, spleen and liver of this animal are reproduced in Plate 2, Figures 1, 2 and 3.

Rabbit 1.—(Full grown Belgian hare, weight not recorded.) On December 13 pricked the conjunctiva of the upper lid of the right eye with a fine needle dipped into the spleen pulp of Guinea-Pig 2. On the third day thereafter the eye was seen to discharge an abundant purulent exudate and the upper lid was congested and edematous. On the fourth day the eyelids which were glued together were separated and the pus washed out with physiologic salt solution. The conjunctiva of the much-congested edematous upper lid showed four discrete yellowish-white areas of necrosis, 2 to 3 mm. in diameter, from which the necrotic material could not be removed by rubbing with a cotton swab. Death occurred during the night of the sixth day.

Post-mortem Examination: The right eye showed considerable congestion and swelling of both lids; the upper and lower palpebral conjunctivae showed a number of pale yellowish-white, irregularly contoured areas of necrosis which were raised above the level of the mucosa, giving the appearance of an adherent membrane; the subcutaneous tissues along the course of the jugular vein on the same side were congested and the lymph-nodes here were enlarged and presented congested capsules. One of the glands was about 8 by 4 mm. in size and on section was of a hard, fibrous consistency, and the cut surface appeared whitish; there was no free fluid in the thoracic and abdominal cavities; the spleen and liver were greatly enlarged, congested, of a firm consistency and full of yellowish-white areas, irregular or nodular, varying from 0.5 to 1 to 2 mm. in diameter; the intestines and abdominal glands appeared normal; the kidneys were congested; the vessels of the heart were deeply injected, and the lungs showed numerous patchy areas of congestion.

The three white rats were inoculated intraperitoneally with guinea-pig spleen extracts and died in two days or less, showing marked injection of the subcutaneous tissues and congestion of the liver, and such congestion of the spleen as to give it a blackish color. In one instance there was also present a complete double pneumonia.

The kittens, which weighed about 400 gm. each, received guinea-pig spleen juice by the ocular, cutaneous and subcutaneous methods, but remained well during several weeks' observation.

The pigeon was inoculated subcutaneously with an emulsion of guinea-pig spleen. It survived during two months' observation, but as the control showed that the virus was dead (48 hours old), this experiment was worthless.

Some experiments showed that death might occur when infectious material is simply placed on the uninjured mucous membrane of the eye or nose, although the first experiment yielded negative results:

On the fourth day after the inoculation of Rabbit 1 some pus was taken from its eye on a cotton swab and rubbed into the left conjunctiva of Guinea-Pig 8. The eye appeared perfectly normal for three days when it was pricked with a needle dipped in the spleen of the same rabbit. In twenty-four hours infection was manifest and death occurred on the fifth day. The post mortem showed characteristic lesions throughout and very similar to those described above for Rabbit 1.

A few drops of an emulsion from the spleen of Guinea-Pig 33, dead of the disease, were allowed to fall into the left eye of Guinea-Pig 38; the latter died in four days, and at post mortem multiple small discrete areas of necrosis were present in the upper and lower conjunctivae; the jugular glands on the corresponding side of the neck were involved and the lesions in the spleen and liver were typical.

A few drops of the same emulsion from Guinea-Pig 33 were allowed to fall into the left nostril of Guinea-Pig 37; the latter was found dead on the third day thereafter; there was a purulent exudate in the left eye, but no ulcers; the jugular glands, spleen and liver showed characteristic lesions.

Single experiments on transmission by feeding and by cohabitation resulted positively in the former case and negatively in the latter:

Guinea-Pig 39 ate most of the spleen of Guinea-Pig 33, which was chopped up and mixed with bread. It died in three days and showed characteristic changes in the liver and spleen and involvement of the cervical glands. The lymph follicles in the small and large intestines showed marked congestion and hypertrophy.

Guinea-Pig 36 was placed in the cage containing Guinea-Pig 35, which was ill of the disease. The latter died one day later, so No. 36 was transferred to the cage containing No. 41, which was ill of the disease. In two days No. 41 died and was allowed to remain in the cage with No. 36 for three days, when the latter was transferred to a fresh cage. Eleven days later No. 36 died without developing any characteristic lesions.

It will be seen that our experimental inoculations, as far as they go, correspond with the findings of McCoy and Chapin. They found guinea-pigs, rabbits, white rats, gray mice, ground-squirrels (*C. beecheyi*), the gopher (*T. bottai*), the Java and Rhesus monkeys quite susceptible; while the adult Norway rat (*M. Norvegicus*) was usually immune; the young were only partly resistant; sheep were only slightly susceptible (one out of five). The following animals they found immune: calf, pig, goat, cat, dog, pigeon. They were able to transmit the disease to guinea-pigs and ground-squirrels by feeding and by nasal inoculation. They found that 0.000001 c.c. of guinea-pig's blood was sufficient to convey the disease to another guinea-pig. They found that the disease was not transmitted by contact or association under the conditions of their experiments, but in searching for a probable mode of natural transmission they twice successfully transmitted the disease by means of 100 and 500 squirrel fleas (*Ceratophyllus acutus*), respectively. They found that the virus in tissues on ice dies in four or five days, and when dried in the incubator for forty-eight hours. We noted about the same degree of longevity on ice and found the virus innocuous when dried at room temperature over calcium chlorid. McCoy and Chapin showed that the disease was not related to plague, for a guinea-pig immune to a virulent culture of *B. pestis* promptly succumbed to this virus.

SUMMARY AND CONCLUSIONS

A case of ulcerative conjunctivitis and lymphadenitis in man is shown to be caused by a minute, capsulated bacterium in all probability identical with *B. tularense,* which was first discovered by McCoy and Chapin in a plague-like disease of the California ground-squirrel (*Citellus beecheyi* Richardson). Without the employment of very special methods of staining and cultivation, the virus, although it was known not to pass the Berkefeld filter No. 5, remained invisible. We recommend anilin-water-Hoffman's violet particularly for its demonstration, and in addition to the coagulated hen's egg-yolk recommended by McCoy and Chapin for its cultivation, we find that hen's ovomucoid with a trace of yolk is also a favorable medium. Our findings would seem to indicate that this disease is wide-spread among rodents. Further, we wish to call attention to the fact that this recently discovered disease of rodents is apparently sufficiently virulent for gray mice (*Mus musculus*) to warrant the presumption that it may some day take its place along with *B. pestis* as a menace to man.

EXPLANATION OF PLATE 3

Figs. 1, 2 and 3 show the gross appearance of the conjunctivae, liver, and spleen of Rabbit 1. Figures 4 and 5 show the spleen and hard, white, inguinal bubo (surrounding congestion not well preserved) of a guinea-pig. All figures natural size. For descriptions see text.

Fig. 6.—Smear from the spleen of a guinea-pig which was chloroformed just before death. It shows both the rod and coccoid forms surrounded by the capsular halo. × 2000. Anilin Hoffman's violet.

Fig. 7.—Smear from a 3-day-old culture of *B. tularense* on coagulated egg-yolk. Anilin fuchsin. × 1000. Photomicrographs by Dr. Chas. Goosmann.

Plate 3

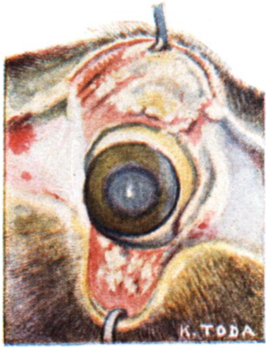
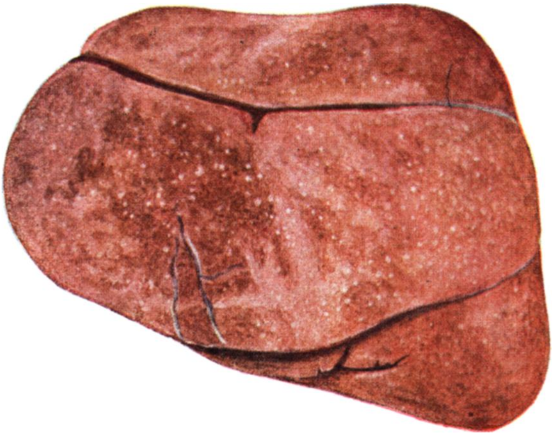


Figure 1



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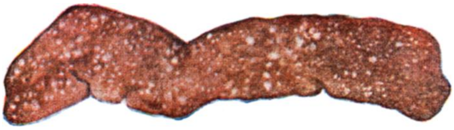

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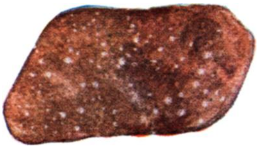

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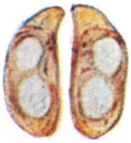

Figure 5

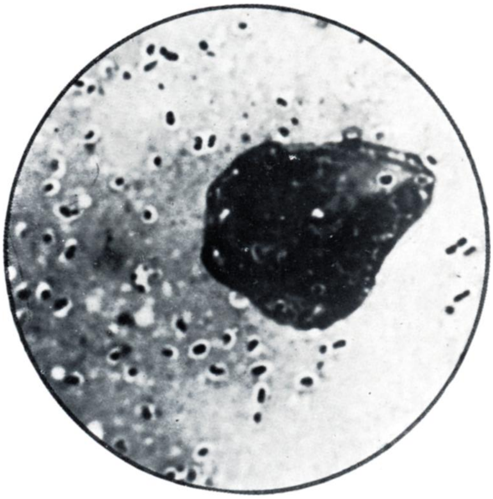

Figure 6

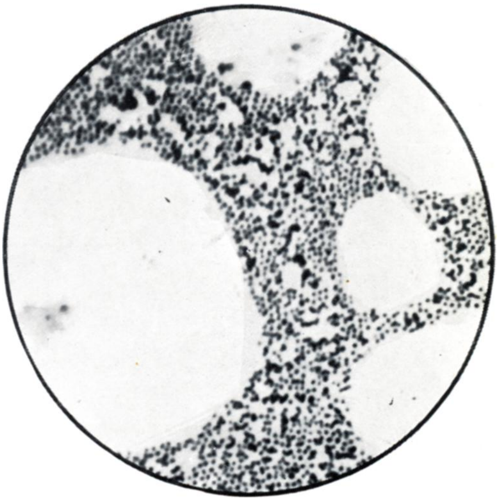

Figure 7